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Effects of bile salts on glucosylceramide containing membranes

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ABSTRACT

The glycolipid transfer protein (GLTP) is capable of transporting glycolipids from a donor membrane, through the aqueous environment, to an acceptor membrane. The GLTP mediated glycolipid transfer from sphingomyelin membranes is very slow. In contrast, the transfer is fast from membranes composed of phosphatidylcholine. The lateral glycolipid membrane organization is known to be driven by their tendency to mix non-randomly with different membrane lipids. Consequently, the properties of the membrane lipids surrounding the glycolipids play an important role in the ability of GLTP to bind and transfer its substrates. Since GLTP transfer of glycolipids is almost nonexistent from sphingomyelin membranes, we have used this exceptionality to investigate if membrane intercalators can alter the membrane packing and induce glycolipid transfer. We found that the bile salts cholate, deoxycholate, taurocholate and taurodeoxycholate, cause glucosylceramide to become transferrable by GLTP. Other compounds, such as single chain lipids, ceramide and nonionic surfactants, that have membrane-perturbing effects, did not affect the transfer capability of GLTP. We speculate that the strong hydrogen bonding network formed in the interfacial region of glycosphingolipid–sphingomyelin membranes is disrupted by the membrane partition of the bile salts causing the glycosphingolipid to become transferrable.

1. Introduction

It is thought that bile salts orient with their sterol nuclei parallel to the membrane surface allowing their hydrophilic groups to form hydrogen bonds with the interfacial water molecules [1–3]. The electrostatic forces between the polar interface and the partially hydrophobic moieties of the bile salt lead to a reduction in the intermolecular hydrogen bonding capacity of the other lipid components in the system [4,5]. Glycosphingolipids also possess both hydrogen donor and acceptor ability with their ceramide backbone, as well as additional hydrogen bonding capability due to their sugar moieties. The high gel-to-liquid crystalline phase melting temperatures of glycosphingolipids are in fact largely due to their ability to form extensive hydrogen bonding networks [6]. Little is known about the connection between bile salts and glycosphingolipids. Two different membrane component classes that both have the ability to form strong hydrogen bonding networks could potentially affect each other's function.

A cytosolic protein called glycolipid transfer protein (GLTP) is able to move glycosphingolipids in vitro between membrane interfaces with its lipid binding domain, shielding the hydrophobic part of the lipids from the aqueous environment. Its unique all-alpha protein-fold has defined a new superfamily [7–10].[1] Another member in the family is the phosphoinositol 4-phosphate adaptor protein-2 (FAPP2). FAPP2 contains a GLTP-like domain, and a pleckstrin homology domain and has been strongly linked to glucosylceramide trafficking [11,12]. Previous in vitro work with GLTP clearly shows that transfer of glycosphingolipids from tightly packed environments, such as different sphingomyelin containing membranes, is very slow compared to membranes containing other acyl chain matched phospholipids [13–15]. How the glycosphingolipid is organized in the membrane laterally therefore becomes important for GLTP transfer efficiency. The membrane organization of glycosphingolipids is driven by their tendency to mix non-randomly with different membrane lipids [16,17]. The clustering of glycosphingolipids together with sphingomyelin and cholesterol in biological membranes is the foundation for the lipid raft concept [18]. Recently we also showed, using a surface plasmon resonance approach, that GLTP still prefers to interact with more tightly packed sphingomyelin membranes compared to chain matched phosphatidylcholines [19], however GLTP-mediated transfer of glucosylceramide (GlcCer) was slower from these membranes compared to phosphatidylcholine membranes [19]. Lateral diffusion of either the glycosphingolipid or the transfer protein or both is therefore required for a protein–lipid complex to form at the membrane interface. The formation of locally concentrated glycosphingolipid clusters is thought to increase the probability of GLTP to associate with a glycosphingolipid [14,20,21], such as if the glycosphingolipid would be in a fluid POPC environment where glycosphingolipids are poorly miscible [22]. If the glycosphingolipid would be well dispersed and partitioned in the bulk membrane lipid phase, such as with sphingomyelins, this would hamper the ability of GLTP to find

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its substrate and hence the transfer rates would be low. Unsaturated phospholipids and glycosphingolipids are not very miscible due to the difference in their acyl chain mismatch. This results in the formation of large lateral glycosphingolipid domains and gives rise to sharp phase boundaries. The difference in T_m between the saturated phospholipids and glycosphingolipids is smaller giving a better miscibility and formation of less segregated domains, and consequently less phase boundaries [16].

Since GLTP transfer activity is depending on the availability and organization of the glycosphingolipids in the membrane, it has prompted us to investigate the influence of different membrane intercalators. These are compounds that could affect the tightly packed sphingomyelin membrane, reorganizing the glycosphingolipid laterally and allowing it to be transferred by GLTP. Consequently, GLTP can be used as a tool to probe the lateral organization of glycosphingolipids in different types of membranes.

Bile salt monomers aggregate to form micelles when their concentrations exceed the critical micellar concentration, CMC. Bile salts have long been known to partition into membranes and alter the membrane properties, such as fluidity and permeability to small ions [23]. Hydrophobic bile salts, common in humans, partitions strongly into membranes and become immediately harmful, because they disrupt the membrane order and impair membrane bound processes [24]. In contrast, hydrophilic bile salts are not so aggressive and even have the capacity to protect hepatocytes from the action of their hydrophobic homologues [25]. Non-conjugated bile salts are located more in the hydrophobic part of the phospholipid membranes, while conjugated more polar bile salts are retained to the more polar interfacial part [26]. Many membrane bound processes require bile salts for their optimal function. For instance, alkaline sphingomyelinase requires bile salts for optimal hydrolytic activity [27] and although most bile salts have a stimulatory effect, the degree of activation varies greatly among bile salt species [28].

To understand the relationship between bile salts and glycosphingolipids better we have examined the influence of different bile salts at submicellar concentrations on the transfer activity of the glycolipid transfer protein. This is to our knowledge, the first study that addresses the relationship between bile salts and simple glycosphingolipids. We have measured how bile salts affect transfer rates of fluorescently labeled BODIPY-glucosylceramide between different phospholipid model membranes. Here we show that the bile salts, cholate (CA), deoxycholate (DCA), taurocholate (TCA) and taurodeoxycholate (THCA) have the capability to clearly alter the packing of tight membranes, and that this also has effects on how glycosphingolipids are organized, at least when probed by the GLTP. We speculate that the strong hydrogen-bonding network formed in the interfacial region of glycosphingolipid–sphingomyelin membranes is disrupted by the membrane partition of the bile salts. Other compounds, such as nonionic surfactants, single and two chain lipids, that also perturb the tight glycosphingolipid–sphingomyelin interaction, but more in the acyl chain region of the membrane, were not able to induce GLTP mediated transfer.

2. Materials and methods

2.1. Materials

1-hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine (POPC), 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (14:0 Lyso-PC), D-*erythro*-sphingosine-phosphocholine (Lyso-SM), hexadecanoyl-ceramide, (d18:1/16:0) N-palmitoyl-D-*erythro*-sphingosine, D-glucosyl-ß-1,1′ *N*-lauroyl-D-*erythro*-sphingosine (12:0-GlcCer), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were all from Avanti Polar Lipids (Alabaster, Al, USA). *N*-(hexadecanoyl)-sphing-4-enine-1-phosphocholine (PSM) was purified from egg yolk sphingomyelin (Avanti Polar Lipids) by reverse-phase HPLC (Supelco Discovery C18-column; dimensions 250×21.2 mm, 5 μm particle size) using 100% methanol as eluent. The fluorescent probes N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)sphingosyl 1-β-D-glucopyranoside (BODIPY-GlcCer C_{12}) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI-C_{18}) were both from Invitrogen (Eugene, OR, USA). Sodium cholate (CA, 5β-Cholanic acid-3α, 7α, 12α-triol sodium salt), sodium deoxycholate (DCA, 5β-cholanic acid-3α, 12α-diol sodium salt), sodium taurocholate (TCA, 5β-cholanic acid-3α, 12α-diol N-(2-sulphoethyl)-amid sodium salt), and sodium taurodeoxycholate (TDCA, 5β-cholanic acid-3α, 12α-diol N-(2-sulphoethyl)-amide sodium salt), were all obtained from Steraloids (Newport, RI, USA). Each bile salt was >95% pure. The nonionic detergents n-octyl-β-D-glucopyranoside (C_8-Gluc), n-dodecyl-β-D-glucopyranoside (C_{12}-Gluc), hexaethylene-glycol-monooctylether (C_8E_6) were all from Affymetrix-Anatrace (Santa Clara, CA, USA) and of ≥97% purity by HPLC analysis. 1-Hexadecanol, palmitic acid (16:0) and dihexadecanoin were from Larodan (Malmö, Sweden). 1-aminohexadecane, hexadecylamine, chlorpromazine (CPZ, 3-(2-chloro-10H-phenothiazin-10-yl)-N,N-dimethyl-propan-1-amine) and Triton X-100 (polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether) were from Sigma-Aldrich (St. Louis, MO, USA). CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) was from MP Biochemicals (Solon, OH, USA).

2.2. Protein expression and purification

The expression and purification of the histidine-tagged bovine GLTP in *Escherichia coli* cells have previously been described [7,29]. Briefly, his-tagged GLTP was transformed in *E.coli*, BL-21 cells, and grown at 37 °C until A_{600} reached 0.6. The expression of the his-GLTP proteins was induced with addition of isopropyl 1-thio-β-D-galactopyranoside (IPTG) and the bacteria were grown at 37 °C for additional 2 h. The bacteria were lysed and purified on a TALON metal affinity resin (BD Biosciences Clontech, Mountain View, CA) according to the manufacturer's instructions. The protein concentration was determined with the method of Lowry [30].

2.3. Preparation of phospholipid vesicles by probe sonication

The lipid stock solutions (in hexane:2-propanol, 3:2) were mixed in desired proportions and dried under nitrogen and redissolved in a 10 mM sodium dihydrogen phosphate buffer, containing 1 mM DTT, 140 mM NaCl and 1 mM EDTA (pH 7.4). The suspension was vortexed and sonicated using a Branson 250 titanium probe (micro tip with a diameter of 3 mm) sonifier and centrifuged for 15 min at 15000 g to remove multilamellar aggregates and titanium probe particles. The size of the vesicles has previously been determined to be about 45 nm in diameter using light scattering measurements with Malvern Zetasizer Nano-S ZEN1600, Malvern Instruments (Worcestershire, UK) [29]. The POPC acceptor vesicles were prepared by sonication. In the transfer assay, the POPC acceptor vesicles (100% POPC) were in a 10 time higher molar concentration (400 nmol) compared to donor vesicles (40 nmol).

2.4. Critical micelle concentration measurements

Typically, ammonium 8-anilino-I-naphthalenesulphonate (ANS) is used as a fluorescent probe for studies of biological membranes and protein structure, however it has also been used to determine the CMC of surfactants [31–33]. ANS is almost non-fluorescent in water, but it fluoresces (λ_{em} 490 nm) strongly when solubilized in a region of lower polarity such as the micelle–water interface. Thus, the CMC can be determined by monitoring an increase in the fluorescence intensity of ANS as a function of surfactant concentration. To a solution of 20 mM NaH_2PO_4, 140 mM NaCl, 1 mM DTT, 1 mM EDTA (pH = 7,4) containing 10 μM ANS, bile salts were stepwise added (2 μl) under

rapid stirring at 37 °C. The emission at 490 nm (λ_{ex} 370 nm) was recorded as a function of the increase in detergent concentration. Two straight lines with distinctly different slopes can be drawn between the measured points and their intersection is taken as the CMC [33]. The CMC values for the bile salts used in this study are presented in Table 1. The CMC values for octyl-glucopyranoside, dodecyl-glucopyranoside and hexaetylenglycol monooctyl ether were also done with the ANS method and presented in Table 2.

2.5. Fluorescence transfer assay

The donor vesicles were composed of 1 mol% BODIPY-GlcCer, 3 mol% of the nontransferable fluorescence quencher DiI-C_{18} and POPC or PSM. A quartz cuvette containing sodium phosphate buffer (total assay volume 3.0 ml) with the bile salt at 0.5 mM was allowed to equilibrate at 37 °C under stirring. Donor vesicles in 100 μl (40 nmol final concentration) sodium phosphate buffer were added to the assay buffer containing bile salts and were allowed to equilibrate for 3 min. Additionally acceptor vesicles in 40 μl (400 nmol final concentration) were added and allowed to equilibrate. The transfer assay was started by addition of 3 μg (40 nM final concentration) of GLTP. The transfer rates during the first minute after GLTP injection, termed initial transfer rate, can be calculated by comparing the increase in the fluorescence intensity (BODIPY-GlcCer unquenching) and comparing this value to the total fluorescence intensity obtained after adding Triton X-100 (final concentration 1%) subtracted with a Triton X-100 blank. A more detailed explanation of the calculations is presented in previously published work [14,20]. In our assay we do not see any increase in the fluorescence intensity without addition of GLTP (data not shown). This is proof that no spontaneous BODIPY-GlcCer transfer occurs, or that any other BODIPY-GlcCer extraction from the donor vesicles occurs. Each experiment (donor vesicle preparation) was repeated at least three times, and at least three transfer measurements were done per experiment. The data points are averages of all transfer rates measurements.

2.6. Steady-state fluorescence anisotropy measurements

Multilamellar vesicles were prepared from pure PSM, 12:0-GlcCer or DPPC (as a control) with diphenylhexatriene (DPH) at 0.5 mol% as a reporter molecule. The lipid mixtures were dried in vacuum for 30 min, resuspended in 2 ml sodium phosphate buffer with 0.5 mM bile salt or the other tested compounds and heated above the transition temperature of the particular lipid. The warm samples were bath sonicated (FinnSonic, Lahti, Finland) for 2 min. The samples containing the fluorescent probe were protected from light during all steps. Excitation was carried out at 360 nm and the emission was recorded at 430 nm as the temperature was scanned from 5 °C to 80 °C at a speed of 5 °C/min using a PTI QuantaMaster 1 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ, USA). The steady-state anisotropy, *r*, was determined as described in Lakowicz [34].

Table 1
Bile salt critical micellar concentrations.

Bile salt	3-OH	7-OH	12-OH	Type/hydrophobicity	CMC (mM)
Cholate, CA	α-OH	α-OH	α-OH	Primary/hydrophilic	5.4
Deoxycholate, DCA	α-OH	H	α-OH	Secondary/hydrophobic	2.5
Taurocholate, TCA	α-OH	α-OH	α-OH	Primary/hydrophilic	4.0
Taurodeoxycholate, TDCA	α-OH	H	α-OH	Secondary/hydrophobic	1.2

Measured with ANS fluorescence in a 20 mM NaH2PO4, 1 mM DTT, 1 mM EDTA, 140 mM NaCl pH = 7.4 buffer.

Table 2
Critical micellar concentrations (CMC).

Detergent	CMC (mM)
n-octyl-β-D-glucopyranoside, C_8-Gluc	20.6
n-dodecyl-β-D-glucopyranoside, C_{12}-Gluc	1.85
hexaethyleneglycol monooctylether, C_8E_6	4.7

Measured with ANS fluorescence in a 20 mM NaH_2PO_4, 1 mM DTT, 1 mM EDTA, 140 mM NaCl pH = 7.4 buffer.

3. Results and discussion

GLTP is not able to transfer BODIPY-GlcCer efficiently from PSM membranes, whereas BODIPY-GlcCer is easily transferred from both POPC (fluid) and DPPC (gel like) membranes [13,14,35]. This finding was used to explore whether membrane intercalators, such as bile salts could affect the tight packing of palmitoyl sphingomyelin (PSM) donor vesicles, allowing GLTP to be able to bind and transfer BODIPY-GlcCer. GLTP would here serve as a selective sensor for membrane environmental changes.

3.1. Bile salt effects on the BODIPY-GlcCer transfer by GLTP

In an initial control experiment we analyzed the effect of four bile salts (Fig. 1A) at 0.5 mM on the transfer of 1 mol% BODIPY-GlcCer from fluid POPC donor to POPC acceptor vesicles mediated by GLTP at 37 °C. As demonstrated in Fig. 1A, GLTP is capable of transferring the BODIPY-labeled GlcCer without any influence of the bile salts. This also shows that the bile salts did not affect the GLTP protein, at least not its transfer capacity. The used concentration of the bile salts (0.5 mM) is well below their CMC values, Table 1. At these concentrations the bile salts do not form micelles, however it is not known if bile salts partition into membranes as monomers or dimers [36]. Bile salts localize in the nonpolar bulk hydrocarbon region and at the membrane interface with their nonpolar moieties oriented toward the nonpolar acyl region and their polar moieties oriented toward the water interphase. Due to their difference in hydrophobicity, bile salts with two hydroxyl groups, DCA and TDCA, are known to partition deeper into lipid membranes than trihydroxy bile salts, CA and TCA [37].

If the donor vesicles consist of PSM, the transfer rate of BODIPY-GlcCer is very low, almost undetectable (Fig. 1B, CTRL bar). When different bile salts were added to the vesicle mixture and allowed to equilibrate, followed by an addition of GLTP, we could observe a very fast transfer rate of BODIPY-GlcCer also from PSM donor vesicles. This rapid transfer is seen for all analyzed bile salts, Fig. 1B. DCA appears to be slightly more effective than CA, but there seems to be no difference between TCA and TDCA. Bile salts with a high degree of membrane partitioning also have a low hydrophilic/hydrophobic balance and a low CMC [38,39]. Deoxycholate with a low hydrophilic/hydrophobic balance partition into membranes more effectively compared to cholate, which can be explained by the absence of the C-7 hydroxyl group in deoxycholate [40]. Introduction of bile salts into the interfacial region of the PSM membrane presumably causes a lateral reorganization of BODIPY-GlcCer, allowing GLTP to bind and transfer its substrate, discussed later in more detail.

We have previously excluded the possibility that the observed decrease in the transfer of glycolipids from sphingomyelin environments was caused simply by phase-transition-related lipid reorganization within the donor mixtures [13,14]. Glycolipids are transferred by GLTP from vesicles composed of phase state and acyl chain matched phosphoglycerides, such as DMPC and DPPC [13,14],

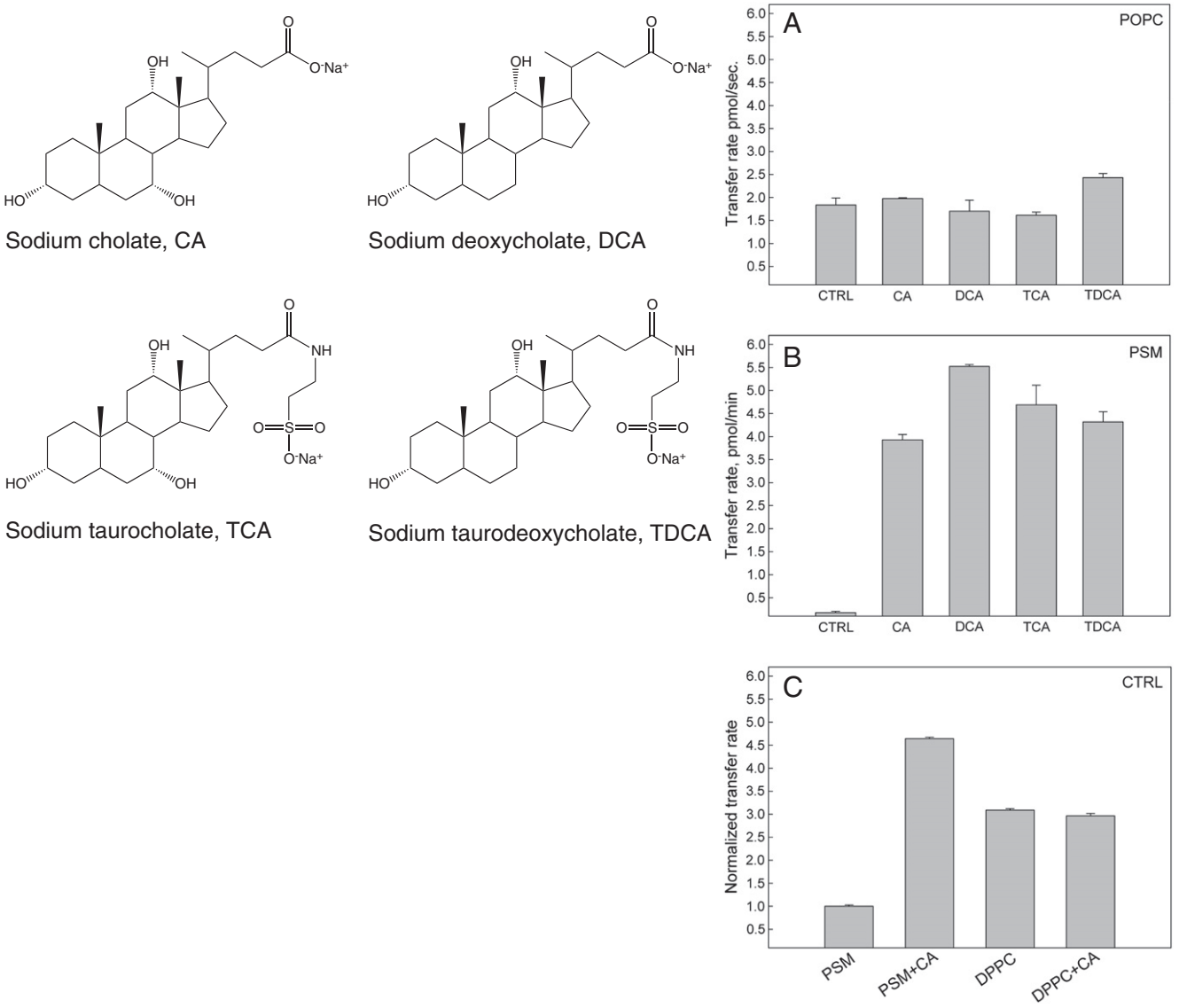

Fig. 1. Structures and the influence of different bile salts (0.5 mM) on GLTP (3 μg) mediated transfer of BODIPY-GlcCer (1 mol%). (A) Transfer rates for BODIPY-GlcCer from POPC donors to POPC acceptor vesicles at 37 °C. The bar marked CTRL correspond to the BODIPY-GlcCer transfer rate from POPC donors with no bile salt added. (B) The influence of different bile salts on GLTP mediated transfer of BODIPY-GlcCer from PSM donor vesicles to POPC acceptor vesicles. The bar marked CTRL corresponds to the very low BODIPY-GlcCer transfer rate from PSM donors with no bile salt added. (C) Control experiment for normalized BODIPY-GlcCer transfer from PSM and PSM with CA compared to BODIPY-GlcCer transfer from DPPC and DPPC with CA. Each experiment was repeated at least three times and the transfer rates are averages of at least triplicate transfer measurements per experiment.

Fig. 1C. In a control experiment we show that the increase in GlcCer transfer from PSM vesicles caused by the addition of the bile salt is not simply a matrix lipid fluidizing effect. Bile salt does not have any effect on the transfer of GlcCer from DPPC membranes containing bile salt (Fig. 1C).

3.2. Effects on the BODIPY-GlcCer transfer of GLTP by other membrane-perturbing agents (long chain alcohols, single- and double chain lipids)

Two compounds that have previously been shown to affect sphingomyelin packing are the long chain alcohol hexadecanol, and its amide counterpart hexadecylamine [41,42]. They partition well between the acyl chains of saturated phospholipids [41,42]. We chose therefore to examine if these single chain membrane intercalators with small polar head groups also could induce GLTP-mediated transfer of BODIPY-GlcCer from PSM donor membranes. These compounds were included together with the lipids for the donor vesicles, rehydrated and probe sonicated. We examined three different concentrations (2, 5 and 10 mol% in the donor vesicles) but could not detect any transfer of BODIPY-GlcCer from PSM membranes (Fig. 2 upper panel). In Fig. 2 we present only the data for 10 mol% of the three single chain compounds, hexadecanol, hexadecylamine and palmitic acid. The compounds did not affect the normally fast transfer of BODIPY-GlcCer from POPC donors to pure POPC acceptors (Fig. 2 lower panel).

The single chain lyso-phosphatidylcholine or lyso-sphingomyelin with large choline headgroups did not have any effects on the PSM donors, tested at both 5 and 10 mol%, only the data for 10 mol% shown, Fig. 3. Nor did these two compounds have any effects on the transfer of BODIPY-GlcCer from POPC donors (data not shown).

Saturated two-chain ceramides and diacylglycerols show an effective competition with cholesterol displacing it from ordered cholesterol/phospholipid membrane domains [42–45]. However they were not able to induce any GLTP mediated transfer of BODIPY-GlcCer from PSM donor vesicles, present at 5 or 10 mol%, only the data for 5 mol% shown, Fig. 3. Nor did these compounds have any effects on the transfer of BODIPY-GlcCer from POPC donors (data not shown).

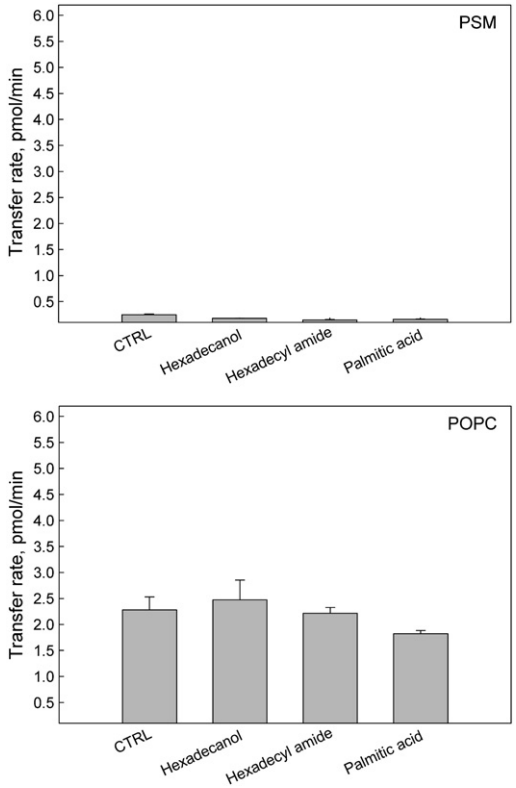

Fig. 2. Effect of the nonionic surfactants hexadecanol, hexadecylamine and the fatty acid palmitic acid at 10 mol% on GLTP (3 μg) mediated transfer of BODIPY-GlcCer from PSM donor to POPC acceptor vesicles at 37 °C. The bar marked CTRL corresponds to the transfer rate from pure PSM donors with no bile salt added. The lower panel shows the transfer of BODIPY-GlcCer from POPC donors to POPC acceptors without (CTRL) and with hexadecanol, hexadecylamine and the fatty acid palmitic acid at 10 mol%. Each experiment was repeated at least three times and the transfer rates are averages of at least triplicate transfer measurements per experiment.

3.3. Effects on the BODIPY-GlcCer transfer of GLTP by nonionic surfactants and CPZ

Alkyl glucosides, common in plants, are nonionic surfactants widely used to solubilize membranes because they partition well into the lipid bilayer at concentrations below their CMC [46,47]. We have here used the synthetic octyl-glucopyranoside (C_8-Gluc) and the longer dodecyl-glucopyranoside (C_{12}-Gluc) below their CMCs (Table 2), to investigate their potential ability to induce a GLTP-mediated transfer of BODIPY-GlcCer from PSM donor vesicles. Another strong detergent, hexaethyleneglycol monooctyl ether (C_8E_6) was also tested (Fig. 4). These experiments were done in a similar fashion as with the bile salts. To a buffer containing the detergents at a 0.5 mM concentration, donor and acceptor vesicles were added and allowed to equilibrate, and the transfer reaction was started with the addition of GLTP. None of these detergents gave any increase in the GLTP mediated BODIPY-GlcCer transfer, Fig. 4. Previous reports have shown that single acyl chain glucosides very weakly interacted with the glycolipid binding site of GLTP [48,49], but in our experiments and at these concentrations we could not see any effects on GLTP.

The addition of donor and acceptor vesicles to CHAPS or Triton X-100 containing buffer caused an immediate increase in the fluorescence intensity, prior to any GLTP addition. We believe that this is a result of a separation of BODIPY-GlcCer from the DiI quencher beyond their Förster distance, indicative of fusion between the donor and acceptor vesicles or vesicles solubilization. Consequently their effects could therefore not be analyzed. Another compound that has been shown to associate strongly to the head group region as well as penetrate into the acyl chain region is the neuroleptic drug chlorpromazine (CPZ) [50–52]. It is thought that CPZ associates well with acidic phospholipids with equimolar stoichiometry and has a significant impact on membrane lateral heterogeneity and rearrangement of the membrane constituents [53]. However, CPZ was not able to induce transfer of BODIPY-GlcCer from PSM vesicles, Fig. 4. The transfer of BODIPY-GlcCer from POPC vesicles was not affected by any of the tested compounds, data not shown.

Based on these experiments it appears that a perturbation of the acyl chain region in PSM membranes caused by the compounds tested in Figs. 2–4 does not alter the BODIPY-GlcCer lateral organization sufficiently so that BODIPY-GlcCer would become available for transfer by GLTP. The lack of the transfer induction effect of hexadecanol and hexadecylamine could be explained by the fact that they intercalate between the acyl chains of saturated phospholipids and induce tighter lateral packing and stabilize the PSM membrane, due to less trans-gauche isomerization, rather than distorting the interfacial region that would weaken the hydrogen bonding network structure. It has also been shown that hexadecanol as well as hexadecylamine displaces sterols from ordered domains and substitute for cholesterol as a spacer between the acyl chains of PSM, leading to an increase in order and a more stabile bilayer [42]. However it appears that a headgroup region perturbation would be required for making BODIPY-GlcCer available for GLTP.

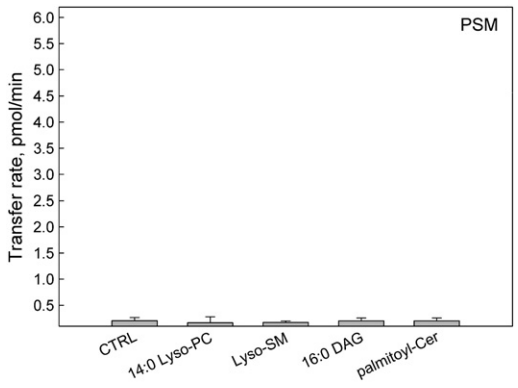

Fig. 3. The influence of lyso-phosphatidylcholine (14:0) and lyso-sphingomyelin at 10 mol% and dipalmitoyl diacylglycerol and palmitoyl ceramide at 5 mol% on GLTP (3 μg) mediated transfer of BODIPY-GlcCer from PSM donor to POPC acceptor vesicles at 37 °C. The bar marked CTRL corresponds to the transfer rate from PSM only. Each experiment was repeated at least three times and the transfer rates are averages of at least triplicate transfer measurements per experiment.

3.4. Effects on the melting temperature of PSM and 12:0-GlcCer by bile salts

How is the packing of the PSM membrane surrounding the GLTP substrate affected by the different surface-active compounds? To analyze the packing properties of PSM and GlcCer bilayer membranes, the steady-state anisotropy of DPH, as a function of temperature was determined. The shorter 12:0-GlcCer was chosen for analysis because of the very high melting temperatures of long chain glucosylceramides [54,55]. The DPH molecule is positioned in the acyl chain region of the lipid bilayer and a higher degree of order for the acyl chains will result in a higher anisotropy for DPH since the possibilities for movement of the DPH molecule are reduced [56–58].

The melting temperatures of PSM and 12:0-GlcCer bilayers containing bile salts, as determined from changes in the steady state anisotropy of DPH, are shown in Figs. 5 and 6. The melting and anisotropy of DPPC served as a control. DPPC and PSM are known to undergo gel-to-liquid crystalline transitions (T_m) around 41 °C [59–61]. As seen in Fig. 5 pure PSM bilayers were highly ordered, based on the measured steady-state DPH anisotropy. After addition of CA and DCA bile salts to

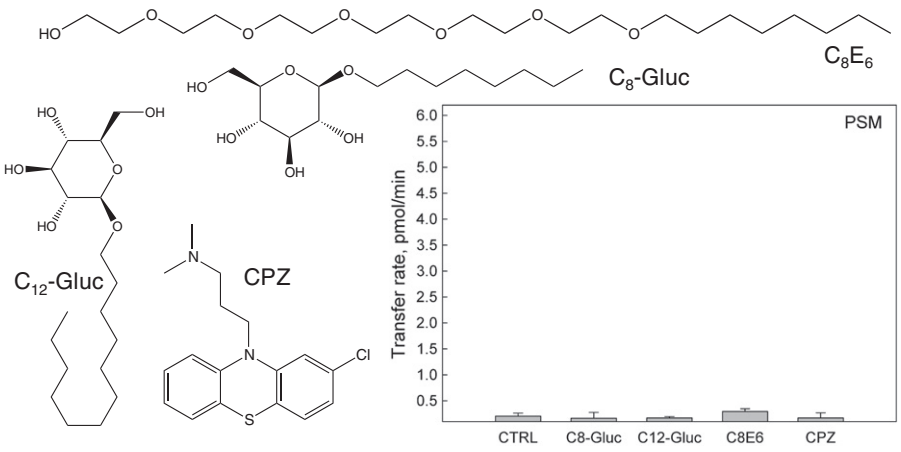

Fig. 4. The influence of octyl-glucopyranoside (C_8-Gluc), dodecyl-glucopyranoside (C_{12}-Gluc), hexaethyleneglycol monooctyl ether (C_8E_8) and chlorpromazine (CPZ) at 0.5 mM on GLTP (3 μg) mediated transfer of BODIPY-GlcCer from PSM donor to POPC acceptor vesicles at 37 °C. The bar marked CTRL corresponds to the transfer rate from PSM only. Each experiment was repeated at least three times and the transfer rates are averages of at least triplicate transfer measurements per experiment.

PSM membranes DPH reported a mild decrease in the overall T_m and anisotropy as the acyl chains of the lipids in the bilayers became more loosely packed. No significant changes in the melting temperature and anisotropy could be seen with the two other tested bile salts (TCA and TDCA). This shows that the PSM membrane is only somewhat affected by bile salts, and that the unconjugated CA and DCA presumable penetrate deeper into the acyl chain region affecting the environment reported by DPH. The conjugated tauro bile salts are more likely to be closer to the interfacial water region [3] and do not to the same extent increase the chain disordering of PSM because of their higher polarity.

The melting temperature of pure 12:0-GlcCer is around 34 °C, according to our DPH measurements, Fig. 6 (GlcCer CTRL bar). Addition of bile salts clearly reduced the melting temperature of 12:0-GlcCer (Fig. 6) as compared to PSM (Fig. 5). The effect is not seen as clearly from the anisotropy data (Fig. 6, lower panel), because 12:0-GlcCer already has a low anisotropy at 37 °C. We speculate that the greater effect of bile salts on the GlcCer melting temperature is a result of the increased disordering of the headgroup region, caused by the weakening of the hydrogen bonding network of the glycosphingolipid with the interfacial water, and perhaps also a reduction in the glycosphingolipid–glycosphingolipid hydrogen bonding capacity.

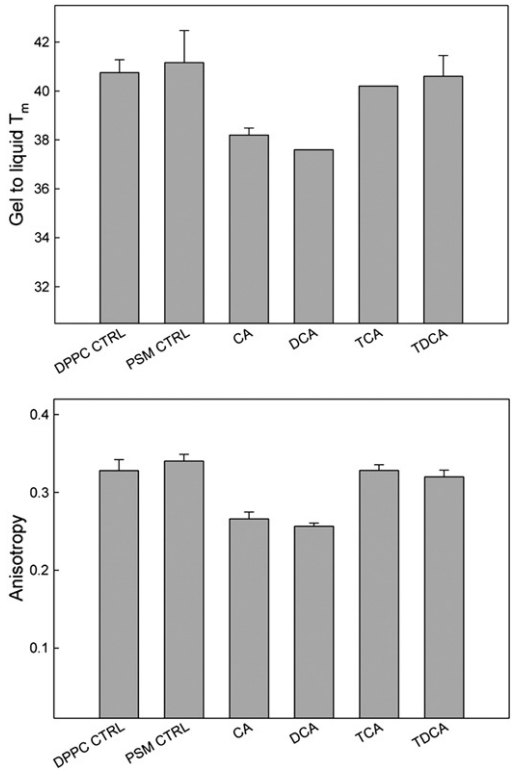

Fig. 5. The influence of bile salts on the gel-to-liquid crystalline melting temperature (T_m) and membrane order of PSM membranes (upper panel), without (PSM CTRL) and with bile salts. The DPPC CTRL bar is the control melting temperature for pure DPPC. The lower panel shows the steady-state anisotropy values of DPH in bilayers at 37 °C degrees for the same mixtures. Each data point is an average of multiple anisotropy measurements.

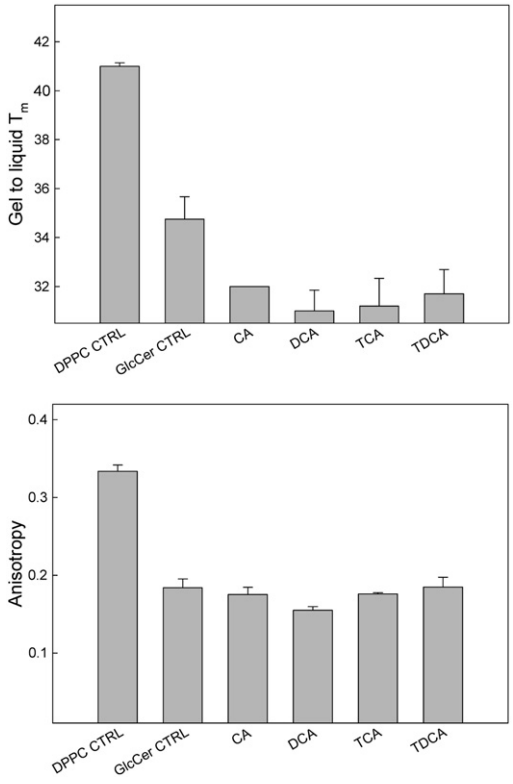

Fig. 6. The influence of bile salts on the gel-to-liquid crystalline melting temperature (T_m) and membrane order of 12-GlcCer membranes (upper panel), without (GlcCer CTRL) and with bile salts. The DPPC CTRL bar is the control melting temperature for pure DPPC. The lower panel shows the steady-state anisotropy values of DPH in bilayers at 37 °C degrees for the same mixtures. Each data point is an average of multiple anisotropy measurements.

This would lead to a lateral reorganization of the glycosphingolipid in such a way that it would become more available for GLTP transfer.

3.5. Effects on the melting temperature of PSM and 12:0-GlcCer by other surface-active compounds

What are the effects of the other used components on the melting behavior of PSM and 12:0-GlcCer? The melting temperatures of PSM (pure PSM melts at 41 °C) and 12:0-GlcCer (pure 12:0-GlcCer melts at 34 °C) bilayers were determined from changes in the steady state anisotropy of DPH and are shown in Fig. 7. The glucopyranosides or the C8E6 detergent did not lower the melting temperature of either PSM or 12:0-GlcCer. As expected hexadecanol [42] and P-Cer [42–45] ordered the PSM membrane further, resulting in an increase in the melting temperature of PSM. Hexadecanol and P-Cer were also able to order the short 12:0-GlcCer. CPZ clearly lowered the melting temperature of PSM. Unfortunately, we were not able to obtain soluble samples with pure 12:0-GlcCer vesicles containing CPZ, and consequently this data could not be analyzed due to the vesicle aggregation. However, none of these compounds was able to affect the transfer of BODIPY-GlcCer from PSM membranes, even though they had effects on the melting of 12:0-GlcCer.

Bile salt monomers partition into membranes differently depending on their hydrophobicity and depending on the nature of the membrane [62]. It is speculated that the difference in the sphingomyelin hydrogen bonding properties compared to phosphatidylcholine would attribute to the difference in bile salt partitioning into these two types of membranes [40]. Glycosphingolipids also possess both hydrogen donor and acceptor ability due to their ceramide backbone, as well as additional hydrogen bonding capability due to their sugar moieties. The high gel-to-liquid crystalline melting temperatures of glycosphingolipids are in fact largely due to their ability to form hydrogen bonding networks through their sugar headgroups [6]. Simple monohexosylceramides like GlcCer and GalCer are able to form two hydrogen bonds each with their eight acceptor oxygens, and in addition six hydrogen donor bonds can be formed with their hydroxyl- and amine groups. This is a great advantage in comparison with membrane phospholipids such as sphingomyelin that has only two hydrogen donor sites and phosphatidylcholine that does not have any at all.

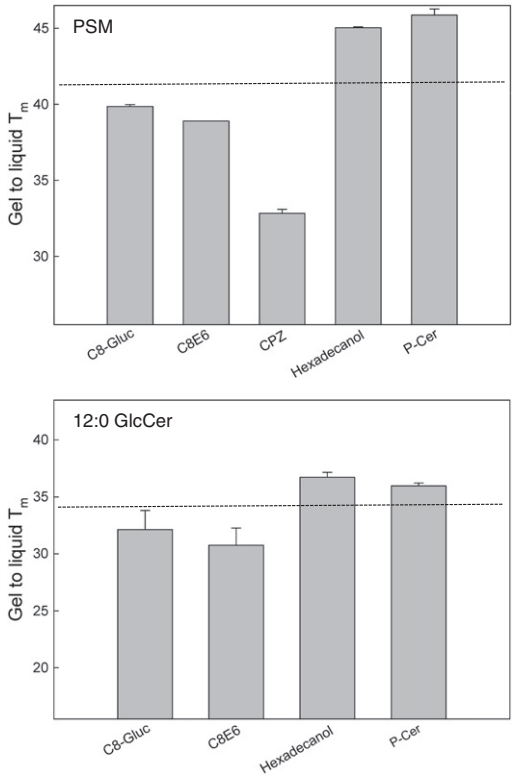

Fig. 7. The influence of different compounds on the gel-to-liquid crystalline melting temperature (T_m) of PSM (upper panel) and 12:0-GlcCer membranes (lower panel). Pure PSM melts at 41 °C and pure 12:0-GlcCer at 34 °C, indicated by the dotted lines. C8-Gluc, CPZ and C_8E_6 at 0.5 mM, hexadecanol and P-Cer at 5 mol%. Each data point is an average of multiple anisotropy measurements.

4. Summary and conclusions

In order to gain better insight into the relationship between glycosphingolipids and bile salts we have utilized the glycolipid transfer protein as a probe. A characteristic feature of glycosphingolipids is their tendency for strong hydrogen bonding networking with molecules in their vicinity, in particular sphingomyelin and cholesterol, and self-association with other glycosphingolipids. This glycosphingolipid "shielding effect" could be of a significant biological importance in lipid raft function and the possible mechanisms for cholesterol selectivity and sorting as suggested by Lingwood and Simons [63]. We have previously speculated that the strong interaction of glycosphingolipids with sphingomyelin would be the cause for GLTP not being able to transfer its substrates from glycosphingolipid–sphingomyelin rich membranes [19,35,64]. If the hydrogen bonding network, and the strong van der Waals interactions in the acyl chain region are broken by surface-active compounds, such as bile salts, the glycosphingolipid lateral organization would be altered and glycosphingolipids would become available for GLTP-mediated transfer.

Bile salts are reabsorbed in the intestine by passive and active mechanisms, bound to proteins, and taken up into the hepatocytes to be reconjugated in the microsomal compartments and secreted again into the bile [65,66]. Bile salts must traverse from one membrane leaflet to the other (flip-flop) in the intestine and liver during recirculation, associate with microsomes during conjugation, and move across the cannilicular membrane during secretion. How this transmembrane movement of bile salts occurs mechanistically and what regulates these events are not well understood [67]. It is tempting to speculate that glycosphingolipids could take part in these events. Here we show that bile salts are capable of affecting glycosphingolipids and their packing and it is therefore also possible that glycosphingolipids could with their hydrogen bonding capacity interact with bile salts on a membrane level and regulate their partition and uptake.

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